BE IT KNOWN that **WE**, Maik **OBENDORF** and Siegmung **WOLF**, citizens of Germany, whose post office addresses and residencies, are, respectively, Paul Schneider Strasse 14, 99423 Weimar, Germany; and Buettelbaum 15, 07639 Bad Klosterlausnitz, Germany; have invented certain new and useful

METHODS FOR DETERMINING HORMONAL EFFECTS OF SUBSTANCES

of which the following is a complete specification thereof:

CROSS-REFERENCE

This disclosure contains subject matter in common with U.S. provisional application, Ser. No. 60/465,692, filed April 25, 2003.

REFERENCE TO SEQUENCE LISTING TABLES

Sequence listing tables are appended hereinbelow listing seven sequences for proteins and nucleic acids. The first sequence listing designated Seq. ID No. 1 for DNA coding for the EWS protein in Seq. ID No. 2. The sequence designated Seq. ID No. 2 is for EWS protein with 656 amino acids. The sequence Seq. ID No. 3 is for synthetic DNA with 20 base pairs, Seq. ID No. 4 is for synthetic DNA with 21 base pairs, Seq. ID No. 5 is for synthetic DNA with 27 base pairs, Seq. ID No. 6 is for synthetic DNA with 33 base pairs and Seq. No. 7 is for synthetic DNA with 18 base pairs.

BACKGROUND OF THE INVENTION

1. Field of the Invention

The invention relates to methods for determining hormonal effects of substances and a method for determining interference in co-modulation mechanisms of nuclear receptors (NR). Further the invention also relates to methods of using Ewing sarcoma protein (EWS) or of EWS derivatives and nucleic acids, which code for them.

2. Description of the Related Art

When substances are judged for possible pharmaceutical applications they are usually tested for contingent hormonal activity, especially for possible androgenic or anti-androgenic activity. Knowledge of those hormonal effects, especially androgenic or anti-androgenic effects, is important for judging possible side effects of administration of these pharmaceutically active substances. For example to test hormonal action of substances methods are used, in which the ability of the substances to bind to hormone receptors and to activate transcription activity is measured.

Knowledge regarding hormonal effects of substances is of interest not only for pharmaceuticals, but also for non-pharmaceutical substances, since many substances in the environment can have androgenic or anti-androgenic or estrogenic or anti-estrogenic activity in part of the population. Possibly undesired injurious effects may occur.

It is especially difficult to identify and characterize effects mediated by steroid hormones, since the signal cascade and networks, which control the hormone mediated transcription regulation, are especially complex. The reason for that is connected with the very similar structure of the DNA target sequences, to which the different steroid hormone receptors bind after ligand activation. This causes the nuclear receptors to turn on a targeted response to interaction with special cofactors, which, among other things, increase the specificity of the receptor-mediated transcription activity.

For identification of substances, which affect certain hormone induced signal paths, thus test systems and methods are required, which can detect the function of individual components of the cellular signal network for mediation of steroidal effects.

There is thus a need for a method, which obtains information regarding the hormonal effects of substances to be tested so that a statement regarding those effects can be made in a reliable, sensitive, simple, economic and rapid manner.

SUMMARY OF THE INVENTION

It is thus an object of the present invention to provide a method for obtaining information regarding the hormonal effects of substances to be tested in a reliable, sensitive, simple, economic and rapid manner.

This object and, others which will be made more apparent hereinafter, is attained by a method for determining hormonal effects of substances, which comprises the following steps:

a) bringing a test substance into contact with Ewing sarcoma protein (EWS) or a derivative of Ewing sarcoma protein and with a nuclear receptor (NR) or a derivative of the nuclear receptor; and

b) determining the effect of the test substance on binding of Ewing sarcoma protein (EWS) or a derivative of it with the nuclear receptor or its derivative; or

c) determining the effect of the test substance on ligand-induced activity of the nuclear receptor.

The term "derivative" of a protein and/or polypeptide (such as EWS) can mean in the context of the present invention any of the following: e.g. variants of the protein and/or polypeptide obtained by amino acid deletion, substitution, insertion, inversion, addition or exchange. Those protein derivatives are especially preferred, which have the ability to influence the activity of other proteins, e.g. of unchanged proteins or polypeptides, or at least to bind to them (functional derivatives).

The invention is based on the surprising knowledge that Ewing sarcoma protein and derivatives of it (henceforth designated "EWS" in the following description) has the ability to interact with nuclear receptors (and/or their derivatives) and modulate their activity.

The super-family of nuclear receptors(NRs), which includes about 50 different proteins, consists of a group of related transcription factors, which control reaction to certain specific ligands, e.g. hormones, like the transcription of a respective target gene. This family can be subdivided into several subfamilies according to certain characteristics, for example dimerization status, type of ligands or structure of the DNA reacting element (Beato et al., 2000, Human Reproduct. Update 6, 225-236). A characteristic feature of nuclear receptors is the corresponding structures of functional domains (marked A to F). These domains consist of a highly variable, only slightly conservative N-terminal region with an autonomous constitutive activation function (AF-1), a well-conserved DNA-binding domain (DBD), which is responsible for recognition of special DNA-binding elements and consists of two zinc finger motifs, a variable hinge domain

and a multifunctional conserved C-terminal ligand-binding domain (LBD) with a dimerization-dependent and ligand-dependent transactivation function (AF-2). This is followed by a region located at the most remote C-terminal, whose function is not known and which is absent in certain receptors. These receptors are, for example, PR (progesterone receptor), PPAR (peroxisome proliferator-activator receptor) and RXR (retinoid X receptor) (Mangelsdorf & Evans, 1995, Cell 83, 841-850; Robyr et al., 2000, Mol. Endocrinol. 14, 329-347). It was demonstrated for some nuclear receptors (for example AR) that the N-terminal region is able to interact with the C-terminal region (Brinkmann, et al, 1999, J. Steroid Biochem. and Mol. Biol. 69, 307-313). Steroid hormone receptors, such as estrogen receptors (ER), progesterone receptors (PR), glucocorticoid receptors (GR), mineralocorticoid receptors (MR) and androgen receptors (AR) bind steroid ligands, such as the progestins, estrogens, glucocorticoids, mineralocorticoids and androgens, all of which are derived from pregnenolone. The ligand binding to the receptor activates the receptor and controls the expression of the corresponding target genes.

EWS is known as a proto-oncogene of Ewing sarcoma and other neoplasms, such as clear cell sarcoma of tendons and aponeuroses, and of small and round cell desmoplastic intraabdominal tumors and extraskeletal chondrosacroma (Delattre, O., Zucman, J., Plougastel, B., Desmaze, C., Melot, T., Peter, M., Kovar, H., Joubert, I., deJong, P., Roubleau, G. Aurias, A., and Thomas, G., 1992, Nature 359, pp. 162 -165; Zucman, J., Delattre, O., Desmaze, C., Epstein, A.L., Stenman, G., Speleman, F., Fletchers, C.D., Aurias, A., and

Thomas, G., Nature Genet. 4, pp. 341-345 ; Gerald, W.L., Rosai, J. and Ladanyi, M.,1995, Proc. Natl. Acad. Sci. USA 92, pp. 1028-1032; Laballe, Y., Zucman, J., Stenman, G., Kindblom, L.G., Knight, J., Turc-Carel, C., Dockhorn-Dworniszak, B., Mandahl, N., Demaze, C., Peter, M., Aurias, A., Delattre, O., and Thomas, G., 1995, Hum. Mol. Genet. 4, pp.2219-2226). The EWS gene locus is rearranged in all these tumors so that the amino acid end (N-terminus) of the protein is fused with a DNA binding domain of FLI1, ERG1, ATF1 or WT1. This N-terminal end of the fusing protein holds the ESW exons 1-7 or 1-8 or 1-9. If the break point lies between exon 7 and exon 8, the EWS portion of the protein arising by the fusion has no correspondence with the androgen receptor binding domain. In contrast if the break point lies between exon 8 and 9 or 9 and 10, only 5 and/or 20 amino acids of both oncogenic EWS fusion proteins correspond with the EWS portion, which contains the androgen receptor binding domain. Thus the rearranged EWS fusion proteins have lost the ability to bind to the androgen receptors.

During analysis of thymus RNA by means of RT-PCR an EWS variant (EWS1-c) was found in which 17 amino acids are missing. Evidently it is a splice variant, since all necessary consensus sequences were present at the neighboring sites between the introns and exons. The result was a shortening of exon 15 (exon 15b). According to the prior art other splice variants are known. One (Ohno, T., Ouchida, M., Lee, L., Gatalica, Z., Rao, V.N., and Reddy, E.S., 1994, Oncobene 9, pp. 3087-3097) is an about 200-bp-shorter EWS transcript (EWS1-b). It was found in resting lymphocytes or in lymphocytes stimulated by phytohemagglutinin (PHA). Exons 8 and 9 are omitted from the EWS1-b.

Another variant (Melot, T., Dauphinot, L., Sevenet, N., Radvanyi, F., Delattre, O. (2001), Eur. J. Biochem. 268, pp. 3483-3489) contains an additional exon 4' between exons 4 and 5 and is characterized as a brain-specific isoform.

EWS belongs to a group of RNA-binding proteins, which are described as implicated in RNA synthesis and processing. Besides that however only little is known about the physiological function of somatic wild-type EWS. Especially the prior art did not known that EWS has the ability to bind to nuclear receptors (NR) and modulate their activity, whereby it is part of the class of nuclear receptor co-modulators.

An *E.Coli* strain designated *Escherichia Coli* EWS-10 CMX was deposited in the German collection of Microorganisms and Cell Cultures GmbH (DSMZ) under the Nr. DSM 15417 on January 24, 2003. *Escherichia Coli* EWS-10 CMX contains the full length EWS-cDNA, which was used in the method according to the invention.

The so-called co-modulators are a class of proteins, which act as bridging modules between the transcription initiating complex and the nuclear receptors in activation (co-activation) and repression (co-repression)(McKenna, et al, 1999, Endocr. Rev. 20, pp. 321-347). A co-activator must be able to amplify the receptor function and to interact directly with the activation domains of the nuclear receptors in the presence of an agonist. It must also interact with the basal transcription apparatus and subsequently it must not increase the basal transcription activity by itself. Most co-modulators interact with the AF-2 domains of the nuclear receptors with the help of one or more LXXLL-motives (NR-boxes).

However a few co-modulators were described which interact with other NR regions (Ding, et al., 1998, Mol. Endocrinol., 12, pp. 302-313). Furthermore many co-modulators were identified, which interact in similar ways with several different nuclear receptors.

The methods according to the invention can be performed both *in vitro* (also e.g. as purely biochemical or biophysical assays, in solution or in suitable solid matrices, etc) and also partially or entirely in cellular systems. One skilled in the art is knowledgeable regarding these test systems.

Preferably at least one of the method steps of the invention is performed in a cellular system, since the effects of the steroid-mediated transcription activity are produced especially well in the physiological context of cells. Both primary and established eukaryotic cells are especially suitable for use in the method according to the invention. The use of established cell lines permits an especially good reproducibility and economy. In contrast, the use of primary cells largely avoids mutation and clone-selected conditioned cell culture artifacts. Prostate cells, nerve cells, glia cells, fibroblasts, blood cells, osteoblasts, osteoclasts, hepatocytes, epithelial cells or muscle cells are especially suitable.

The hormonal effects determined here (i.e. identification, quantification or characterization) can be both of an activating and also inhibiting nature and can also relate to other steps of nuclear receptor action besides activation on receptor-co-modulator binding, e.g. ligand-induced transactivation and also nuclear localization of the nuclear receptor.

Preferred embodiments of the method include the following steps:

a) first cells, which express EWS or a derivative of it and a nuclear receptor or a derivative of it, are exposed to the test substance;

b) the protein-protein interaction or the protein-protein-DNA interaction is measured to determine the effect of the test substance on the interaction between the receptor or its derivative and EWS or its derivative.

Expression of one or both components interacting with each other (EWS/derivative, on the one hand, and NR/derivative, on the other hand) can occur in cells from nature or as a result of transient or stable transfection with suitable expression vectors. The selection of suitable cell types and if necessary vector systems is a standard procedure known to those skilled in the art. For example pCMX or pSG5 is suitable for expression in eukaryotic systems.

The measurement of protein-protein interaction between receptor and/or its derivatives and EWS or its derivatives or the protein-protein-DNA interaction of the above-described components with DNA target sequences occurs by procedures known to those skilled in the art. For this purpose techniques, such as the two hybrid system, co-immunoprecipitation, GST pull down assays, FRET analyses and ABCD assays and/or gel retardation assays are suitable for analysis of protein-protein-DNA interactions.

In preferred embodiments of the method according to the invention the following steps are performed:

a) cells, which express EWS or a derivative of it and a nuclear receptor or a derivative of it and are transfixed with a reporter gene construct, are exposed to ligands of the nuclear receptor and the substance to be tested;

b) transcription activity of the nuclear receptor is determined by measuring the reporter gene activity; and

c) the transcription activity ascertained by performing steps a) and b) is compared with that when the test substance is not present.

Reporter genes are genes or gene fragments, which code for as simple as possible gene fragments, e.g. photometically by dye reactions. Frequently used reporter genes are the gene for β-galactosidease, the gene for alkali phosphatase, the gene for chloramphenicol-acetyltransferase, the gene for catechol-dioxygenase, the gene for "green" or "blue fluorescent protein" as well as different luciferase genes, which can cause cell luminescence. The activity of the transcription factor and/or the cascade can be determined with the aid of the expressed gene product by a series of suitable control elements, e.g. a promotor-enhancer sequence, which is under control of a certain transcription factor or a certain signal transduction cascade.

This sort of reporter gene is conventionally introduced into the cells in a suitable vector in connection with an interesting promotor-enhancer sequence. All known nuclear receptor target sequences -- depending on the nuclear receptor to be analyzed -- are suitable for analysis of the steroidal activity of substances. For example the MMTV-luciferase vector, which is used for measurement of androgenic activity of substances, is suitable for use as a vector in the method according to the invention.

Substances with a hormonal effect, preferably an androgenic/ anti-androgenic effect, are then detected by an increased or reduced expression of

the reporter gene in comparison to experimental assays without addition of the substance to be tested.

Besides wild type EWS, EWS derivatives, and especially functional EWS derivatives, which have kept the ability to modulate the activity of at least one nuclear receptor, especially the androgen receptor, or at least to bind to it (in a not negligible manner detected by suitable methods -- e.g. protein-protein interaction assays like EMSA; which one skilled in the can differentiate) are suitable in the method according to the invention. The same goes for NR derivatives: Those derivatives are also preferred, which have maintained the ability to be modulated or at least bound by EWS or its functional derivatives.

EWS and EWS-coded nucleic acids are already known in the prior art. Preferably an EWS coded by the nucleic acid according to Seq. ID No. 1, or its derivative (especially a functional derivative), are suitable for use in the method according to the present invention. An EWS derivative, which has amino acids 319 to 656 of the sequence described Seq. ID No. 1, especially a fragment containing these amino acids, is especially preferred.

The invention accordingly relates to the use of ESW or its derivatives for identification and characterization of substances that influence the activity of a nuclear receptor.

Beyond this the present invention relates to the use of nucleic acids with at least 70 % homology to Seq. ID No. 1 or to sequence region 8 to 2032 or sequence region 1000 to 2011 of Seq. ID No. 1 for identification and characterization of substances, which influence the activity of nuclear receptors.

These types of nucleic acids are cloned in expression cassettes of suitable expression vectors, especially eukaryotic expression vectors.

The term "nucleic acids with at least 70 % homology to Seq. ID No. 1" is understood to mean the entire range between 70 % and 100 % homology (also complete correspondence with Seq. ID No. 1). The selection of nucleic acids suitable for the respective purpose in the stated homology range is within the ability of those of ordinary skill in the art. The determination of the nucleic acid homology occurs in a way that is familiar to those of ordinary skill in the art. For this purpose different computer programs, for example BLAST, BLAST-2, ALIGN or MEGALIGN (DNASTAR), are known to those skilled in the art.

The methods according to the invention or the uses of the above-described proteins and/or nucleic acids are suitable especially for analysis of hormonal effects of substances at androgen receptors, estrogen receptors (α and β), progesterone receptors, glucocorticoid receptors, mineralocorticoid receptors, thyroid gland hormone receptors, vitamin-D receptors, peroxisome proliferator-activated receptors, retinic acid receptors, retinoid-X receptors or orphan receptors. Because of the especially good characterizing action of EWS and/or EWS derivatives at the androgen receptor, it is employed in especially preferred embodiments of the method according to the invention.

Beyond this EWS can be used as a clinical indicator of androgen-conditioned illnesses. Relevant androgen-conditioned illnesses include e.g. prostate cancer, baldness, acne or hypogonadism, and androgen-resistant syndromes, such as testicular feminization. These illnesses are probably based

on defects in the co-modulator mechanism between androgen receptor and EWS. Thus measurement of the relative rates of AR and EWS is a plausible diagnostic possibility for patients with this sort of trouble. This is possible using a quantitative measurement method for relative amounts of both molecules in the target tissues in the respective patients.

A further aspect of the invention accordingly relates to the use of a nucleic acid with at least 70 % homology to Seq. ID No. 1, to use of sequence ranges 8 to 2032 or 1000 to 2011 of Seq. ID No. 1 or to use of an antibody, which is directed against a protein coded by these nucleic acids, to diagnose illnesses which accompany a dysfunction of nuclear receptor activity, preferably androgen receptor activity.

One such use advantageously occurs in a method for determining interference with the co-modulation mechanism between androgen receptor and EWS, in which the cellular concentrations or tissue concentrations of androgen receptor and EWS are measured. Especially radio immunoassays, ELISAs, immunodyeing, quantitative RT-PCRs, Northern blot or Western blot are among the different techniques suitable for this purpose known to those skilled in the art.

These sorts of measurements of the relative rates of androgen receptor versus EWS have the theoretical basis that androgen-resistance syndrome is based on an interference of the equilibrium between AR- and EWS-prevalence in the target cells. Too much EWS could lead to an over-sensitivity of the androgen receptor system, so that it reacts to molecules, which normally have no

androgenic effect. Absence of EWS or EWS function can lead to low or reduced sensitivity at all levels of androgen resistance.

Furthermore it is possible to construct a PCR assay with the help of a suitable EWS-cDNA primer, with which mutations of the normal DNA sequence may be detected in certain patients or transcripts for the Northern Blot Assay and/or a DNA for In-situ-hybridization assays may be generated.

The detection of too much EWS in patients speaks for the use of means or measures for lowering the EWS level. This can occur, for example by means of antisense nucleic acids relative to EWS or EWS derivatives or by similar techniques to reduce the EWS titer in the respective patients under clinical conditions. This could also be achieved by molecules, which are in a position to inhibit the interaction between AR and EWS.

In contrast should a patient have too low a level of EWS, one could administer EWS-cDNA, EWS-protein or EWS DNA to him in order to increase the titer of active EWS in this manner. Another aspect of the invention relates accordingly to the use of the above-described EWS or EWS-derivative-coding nucleic acids or EWS proteins or EWS derivatives, which are coded by those nucleic acids, for therapies for illnesses dependent on dysfunction of nuclear receptor activity.

BRIEF DESCRIPTION OF THE SEVERAL VIEWS OF THE DRAWING

The objects, features and advantages of the invention will now be illustrated in more detail with the aid of the following description of the preferred embodiments, with reference to the accompanying figures in which:

Figure 1 is a diagrammatic illustration of the gene for the androgen receptor (AR) and the AR2 fragment, in which the androgen receptor fragment (AR2) is designated as AS: 325 – 919; the activation domain with AF, DNA binding domains, with DBD; ligand binding domains with LBD; activation domains with AD and binding domains with BD;

Figure 2 is a diagrammatic illustration of the gene for the Ewing sarcoma protein (EWS), in which blue shows the RNA binding domain, dark red, the androgen receptor binding domain AS 219 - 656;

Figure 3 shows the amino acid sequence of the EWS protein and the EWS exons coding the EWS protein;

Figure 4 is a graphical illustration showing relative co-activation of the AR signal in SH-SY5Y cells with CMX and EWS respectively; and

Figures 5a and 5b are respective illustrations showing the distribution of EWS transcripts and AR transcripts in various tissues.

EXAMPLES

Example 1:

Oligonucleoties Employed:

Primer for the PCR amplification of library inserts:

Act2c5050Eco: gattacgctagcttgggtgg (Seq. ID Nr. 3)

Act2-4939Xho: gttgaagtgaacttggcgggg (Seq. ID Nr. 4)

Primer for Amplification of EWS-cDNA in full length:

EWS-8-Sal: gggtcgacggacgttgagagaacgagg (SEQ ID Nr. 5)

cESW-c2032-Eco: gggaattctgcggggtctcgcatctagtaggg (SEQ ID Nr. 6)

Sequence Primer:

XII-139a1: gcttgggtggtcatatgg (SEQ ID Nr. 7)

Vectors Used:

pACT2 (Genbank Access Number U29899) for the library;

pGBT9 derivative for the probes: pGBT9rev and pGBT(+1)rev (Roder, K.H.; Wolf, S.S.; Schweizer, M., 1996, Analytical Biochemistry, 241, pp. 260-62);

pCR2.1 Topo-Vector (Invitrogen Co) for coding of the PCR fragment;

CMX Vector for expression of mammalian cells;

PAluc for reporter gene assay (contains the MMTV promoter and a Luciferase reporter gene; A. Cato Co.);

pSG5AR (pSG5 with the human genes for the androgen receptor; Gene bank access number AAA51775).

Organisms used:

Yeast strain:	Y187 and PJ69-2A
E-Coli Strain:	DH5α
Mammalian Cells:	SH-SY5Y (German Collection of Microorganisms and Cell Cultures GmbH (DSMZ): DSM ACC209); PC3 (American Type Culture Collection (ATCC): CRL-1435; and PC3AR: with pSG5AR stabile transfixed PC3 (A. Cato Co., Karlsruhe, Germany)

To identify new co-modulators of the androgen receptor a Human cDNA library ("Matchmaker" of Clonetech; Nr. HY4028AH) from fetal brain was screened with three different fragments of the androgen receptor (AR) as probe with the help of a yeast-two hybrid system.

For this purpose pSG5AR vector, which contains the cDNA for the human androgen receptor (Genbank AAA51775), was cleaved with the help of Endonuclease PstI, so that three different AR-DNA fragments were produced. The shortest of these fragments (AR4) coded for the N-terminus of the receptor (AS 1 – 56), the middle length fragment (AR3) coded for the middle part with the activation domain (AS 57 – 324) and the longest fragment (AR2) coded for the

C-terminus (AS 325-918) with the DNA and ligand binding domains (DBD and LBD; compare with Fig. 1). AR2 was cloned in the pGBT9(+1)rev vector, since it was previously linearized with the help of endonuclease PstI.

Subsequently the transformation of the pGBT vector, which contains the AR fragment, occurs in the yeast strain PJ69-2A. The positive transformant (Trp+) was incubated with a cDNA library obtained from fetal brain according to the instructions from the manufacturer (Human Multiple Tissue cDNA (MTC), Panel II of Clontech Cat. Nr. K1421-1). $3x10^6$ clones were screened in accordance with the instructions from the manufacturer (Clontech). The positive clones were selected and tested for their β-galactosidase activity according to the instructions of the manufacturer (Clontech). The inserts of the blue colonies originating from the library were increased directly from the yeast cells by means of PCR using the primer Act2c5050Eco and Act 2-4939Xho.

The PCR products were further analyzed by gel electrophoresis for its length after scission and by means of cleavage with MspI. At least one example of each restriction fragment pattern was sequenced using XII-139a1 as sequence primer. The sequences were compared with Incyte of Genbank or Databank.

One of the many identified inserts had a length of 1500 bp and could be identified by sequencing and sequence comparison with Databank NCBJ as coding for the C-terminal part of human EWS (AS 319-656) (see fig. 2 and amino acid sequence in fig. 3).

Fig. 3 shows the cDNA sequence of human EWS together with the derived amino acid sequence. Exons 1 to 17 are shown. The letters printed in

bold face characterize the fragment, which is to be found in the yeast two hybrid system and binds to the androgen receptor section AS 325 to AS 919. The sequence regions absent in the splice variant EWS1-b (underlined with a solid line) or EWS1-c (underlined with a dotted line) are underlined in fig. 3.

EWS in its full length was amplified or increased by means of PCR using EWS-8-Sal primer and cEWS-c2032-Eco primer as well as thymus-cDNA or spleen-cDNA of Clontech. The complete coding region of the transcript was isolated from spleen and the variant with exon 15B instead of exon 15 was isolated from thymus. The amplified cDNA was then cloned with EcoRI and Sal I in the expression cassette of mammalian expression vector CMX.

Figure 4 shows the co-activation of the AR signal in SY-SY5Y cells. 1 μg MMTV-Luciferase and 0.75 μg pSG5AR plasmid were supplied to each reaction chamber of a six-reaction-chamber reaction plate. Each of these six mixtures was transferred to four cavities of a microtiter plate and measured there. The error bars show the standard deviation SD. The measured values were obtained by subtracting the corresponding control values without DHT.

As the bar graph shown in fig. 4 shows, after transient transfection in SH-SY5Y cells EWS is able to induce a strong co-activation of the androgen receptor signal action, especially at low androgen concentrations of 10^{-12} to 10^{-10} mol. For this purpose SH-SY5Y cells in reaction plates with six reaction cavities were transfixed with 0.75 μg of a vector, which contained the cDNA for the human androgen receptor (pSG5AR), 1.5 μg of reporter gene construct pAHluc, which contains the MMTV promoter for the Luciferase gene, and 1 μg of EWS-

CMX vector. The transfection occurred using lipofectin of Gibeo BRC according to the instructions of the manufacturer. Twenty-four hours after the transfection the cells were incubated over night with different androgen amounts. The cells were subjected to lysis with a commercial lysis buffer and the luciferase activity was measured in a Lumistar luminometer of BMG Lab Technologies. The EWS-CMX Luciferase activity was compared with the control activity (empty CMX vector). The mixture in each cavity was measured in four cavities of a microtiter plate. The control values of the substance were subtracted without DHT. The standard deviation was shown with vertical lines indicating the range on the bars in fig. 4.

The tissue distribution of human EWS in normal human tissue is shown in fig. 5a with the aid of autoradiography. Tissues numbered 1 to 16 show the relative amount of human androgen receptor in the following tissues respectively: heart, brain, placenta, lung, liver, skeletal muscle, kidney, pancreas, spleen, thymus, prostate, testicles, ovaries, small intestine, large intestine and peripheral leucocytes. For this purpose an EWS-cDNA fragment, which coded for amino acids 244 – 656 of EWS, was marked with ^{32}P-α-dATP and the Klenow fragment, according to the MEGAPRIME® Marking system. The marked fragment was purified with a Nick column (Pharmacia) according to the instructions of the manufacturer and was hybridized with Human Blot and Human Northern Blot (MTN) Nr. 7760-1 and Nr. 7759-1 of Clontech. As shown from figure 5a, EWS-RNA is predominantly expressed in testicles. Different tissues contain different amounts of EWS.

Fig. 5b shows the tissue distribution of human androgen receptor in normal human tissues. Tissues numbered 1 to 16 show the relative amount of human androgen receptor in the following tissues respectively: heart, brain, placenta, lung, liver, skeletal muscle, kidney, pancreas, spleen, thymus, prostate, testicles, ovaries, small intestine, large intestine and peripheral leucocytes. From figs. 5a and 5b one can ascertain the normal expression of both these proteins in tissue.

Figure 5a shows the tissue distribution of EWS transcript (Northern Blot MTN of Clontech). A random priming of the EWS-cDNA fragment of the manufacturer (Amersham), which codes for the amino acids 244 to 656, and marking with ^{32}P-α-dATP and the Klenow fragment took place according to the instructions of the manufacturer. The blots were hybridized with the probe, washed, transferred to a film and developed.

The disclosure in German Patent Application 103 09 280.3 of March 4, 2003 is incorporated here by reference. This German Patent Application describes the invention described hereinabove and claimed in the claims appended hereinbelow and provides the basis for a claim of priority for the instant invention under 35 U.S.C. 119.

While the invention has been illustrated and described as embodied in a method for determination of hormonal effects of substances, it is not intended to be limited to the details shown, since various modifications and changes may be made without departing in any way from the spirit of the present invention.

Without further analysis, the foregoing will so fully reveal the gist of the present invention that others can, by applying current knowledge, readily adapt it for various applications without omitting features that, from the standpoint of prior art, fairly constitute essential characteristics of the generic or specific aspects of this invention.

What is claimed is new and is set forth in the following appended claims.